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ORIGINAL ARTICLE

Group-spawning and Simultanous Polyandry of a Stream-dwelling Frog *Feirana kangxianensis*

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Abstract Here we provide an example of simultaneous polyandry based on genetic evidence in *Feirana kangxianensis*. This stream-dwelling species occurs only in Kangxian County, southern Gansu Province, where it is sympatric with its sibling species *F. quadranus*. During the breeding season the sex ratio of *F. kangxianensis* was marginally female-biased (44♂:59♀) and the encounter rate in a relatively pristine habitat was significantly higher than that in heavily quarried habitats (9.6 ± 4.8 indiv./km *vs.* 3.2 ± 2.5 indiv./km). Three egg masses containing an average of 698 eggs were deposited on the underside of one or two adjacent flat rocks, 6.0–10.0 cm under the water surface and 1.0–3.5 cm above the streambed. Using Bayesian sibship clustering of nine polymorphic microsatellite genotypes, two females were detected as group-spawning in one oviposition site, with three males fertilizing each female's eggs simultaneously. We also discuss the conservation requirements of this range-restricted species and the evolutionary implication of its unusual reproductive strategy.

Keywords group-spawning, oviposition site, sex ratio, simultaneous polyandry, habitat conservation

1. Introduction

Three species in the genus *Feirana* are known from central China and all occur in montane streams at 300–2000 m a.s.l. (Fei *et al.*, 2012). *Feirana quadranus* occurs across the Qinling-Daba mountains with a total range of 290 000 km^2 (29–35°N, 104–112°E) whereas *F. taihangnica* has a smaller range of 30 000 km^2 at higher latitudes (33–36°N, 107–113°E). *F. kangxianensis* is distributed narrowly in Kangxian County (900–1700 m a.s.l.; 33.0692–33.2804° N, 105.3674–105.7756° E) (Yang *et al.*, 2011), where it is sympatric with *F. quadranus*.

Feirana taihangnica is uniquely characterized by the swollen cloaca that occurs on both sexes. They are aggregate breeders and have no amplectant behaviors (Chen *et al.*, 2011; Zhang *et al.*, 2012). In contrast, *F. kangxianensis* has no swollen cloaca but spines on the cloaca, dorsal skin, thumbs, and index fingers in males (Yang *et al.*, 2011). No data are available on the reproductive ecology of this geographically restricted species. Here we describe for the first time the habitat requirements and breeding biology of this poorly known species *F. kangxianensis*.

2. Study area and Methods

2.1 Study area Our study area (33°11'55" N, 105°21'41" E) was the Liuba stream (5–15 m wide, 7 km long, 1600–1800 m a.s.l.) in Kangxian County, southern Gansu, which winds through a valley covered by croplands and mixed deciduous broad-leaved and coniferous trees. Five villages (about 500 families and 2000 villagers) distribute along both sides of the main stream. Nearly all large stones in the stream were quarried for construction purposes after the Wenchuan Earthquake in 2008, so that the runoff was altered and the water depth in the stream

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varies from 0 to 80 cm. The air temperature and water temperature of the stream measured at 14:00 and 21:00 each day during 7–24th April were averaged 13.3 ± 3.5 °C (6.0–19.5) and 17.8 ± 5.2 °C (9.1–28.0), respectively.

2.2 Field survey We set five transects, three in the main stream (A: 4.0 km, B: 0.6 km, C: 2.5 km) and two in tributaries (D: 1.0 km, E: 2.0 km; Figure 1). Transect D is located in a steep, pristine tributary with no adjacent villages. During the 7–24th April, two investigators walked along the stream and carefully inspected rocks larger than 10 cm in diameter during the daytime (08:30–18:00) or at night (20:00–22:00) with torches. Encounter rates were calculated for each census as number of encounters (adults only) divided by the total distance travelled (i.e. survey effort).

For each captured frog, we measured its snout-vent length (SVL) to the nearest 0.1 mm and mass to the nearest 0.1 g, clipped the first segment of one or two toes for DNA sample (Gonser and Collura, 1996; preserved in 95% ethanol), and then released at the site of capture. For each egg mass found, we identified the stages of embryo development and estimated the laying date following the timing of developmental stages in Tao *et al.* (2010). We randomly sampled 56 embryos from different positions in one egg mass (503 eggs in total) to determine the number of mothers and the paternity of offspring.

2.3 Genetic classification of sympatric species Total genomic DNA was extracted from toe tissues or embryos using the EasyPure Genomic DNA Extraction Kit (Beijing TransGen Biotech Co., Ltd.). To differentiate females of *F. kangxianensis* and *F. quadranus*, a portion (701 bp) of the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) was amplified and sequenced following the procedure of Wang *et al.* (2009) using the pair of primers: Pmet (Wang *et al.*, 2009) and ND2mid (5'-GGTCATGATGAGGAGAT-3', designed in this work). All ND2 sequences were aligned with the CLUSTRAL W option and default parameters in MEGA 6.0.6 (Tamura *et al.*, 2013). Bayesian inference was used for species assignment and were performed using MRBAYES version 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist *et al.*, 2012).

2.4 Maternity and paternity of embryos Nine microsatellite loci (CIBfq01, 03–08, 17 and 21) that developed on *F. quadranus* were selected for amplification in all samples according to the procedure of Wang *et al.* (2014). Basic descriptive statistics for each microsatellite locus and the sensibility of these microsatellites to resolve paternity or maternity was determined by using

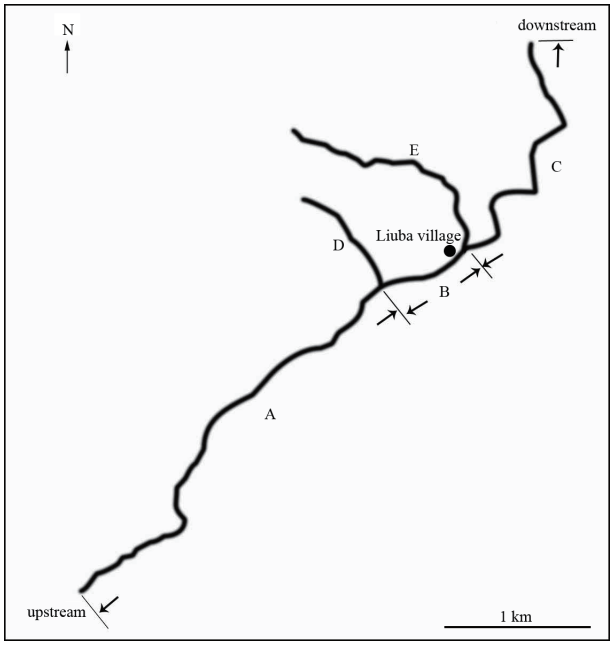

Figure 1 Five transects were set at the Liuba stream, Kangxian County: A (4.0 km), B (0.6 km), C (2.5 km), D (1.0 km), E (2.0 km). D is a pristine tributary without adjacent villages.

Cervus 3.0 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007). Sibships of embryos were reconstructed by using COLONY program (Jones and Wang, 2010), with settings of full-pedigree likelihood method, polygamous mating system, sibship complexity prior, allelic dropout rate of 0.01 and false allele rate of 0.01 for each locus. The same dataset was processed in at least eight runs with different random seeds to confirm the reliability of results. The inferred paternal genotypes were compared with those of candidate parents (i.e. adults captured 0–1000 m from the breeding site) based on the inheritance rule of codominant markers in diploid species. If mismatches were found at two or more loci, an adult was excluded as a potential genetic parent.

3. Results

3.1 Population composition and habitat selection A total of 44 males and 59 females of *F. kangxianensis* were captured between 7th and 24th April. The sex ratio of this sample was female-biased (1:1.3), although the bias was not significant ($\chi^2 = 2.18$, $P = 0.14$). Ignoring the ages of frogs sampled, males were significantly smaller than females (SVL: 9.58 ± 1.07 *vs.* 8.04 ± 0.85 cm; $t = 7.43$, $df = 89$, $P < 0.001$).

The encounter rate of *F. kangxianensis* varied among transects A, B, C, D, and E, and averaged 1.9, 2.8, 6.4, 9.6, and 2.0 indiv./km, respectively (Figure 2).

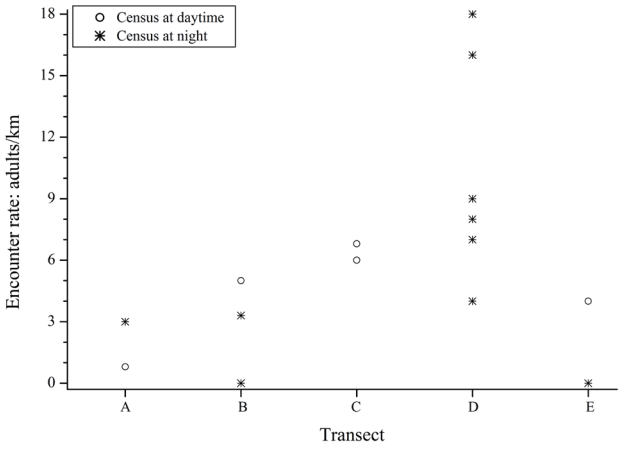

Figure 2 Encounter rate of *F. kangxianensis* in the five transects in the Liuba stream, Kangxian County, Gansu Province. Detailed information of transects is provided in Figure 1.

3.2 Aggregate breeding Frogs may aggregate in the breeding season as we captured seven and nine adults respectively in two pools with diameters of 3–5 m and a depth of 1.8–2.3 m. Four females and five males were found in one bank hole (30 cm × 13 cm × 13 cm) when eggs being laid inside.

Three oviposition sites, with the inter-distance of 6–12 m, were found where the stream was 7.0–8.0 m wide and 9.0–14.0 cm deep at 15:00–16:00 on 17th April. Eggs were arranged in a single horizontal layer beneath one or two adjacent rocks (50–76 cm × 26–36 cm) that were attached to the riverbank (n = 2) or in the middle of the stream (Figure 3). The egg masses (20–50 cm × 15–25 cm) were located 6.0–10.0 cm beneath the water surface and 1.0–3.5 cm above the streambed.

Embryo development stage of the three egg masses (522, 1069, and 503 eggs) were identified as 4-cell to early blastula stage, neural tube stage, and muscular response stage, and thus egg laying was backdated on April 17, 12, and 9th, respectively.

3.3 Mating system Based on the genotypes of nine polymorphic microsatellite loci in 37 females and 35 males, the combined probabilities of non-exclusion for the first and second parent were 1.9×10^{-3} and 6.0×10^{-5} respectively (Table 1), indicating a sufficient power to resolve paternity. Furthermore, these markers were able to discriminate between full siblings with a power of 6.3×10^{-12}.

Using Colony, the 56 sampled embryos were inferred to come from two mothers with 41 and 15 offspring respectively, and from three fathers with 35, 5, and 1 offspring, respectively (Table 2). The dominant male fertilized 45 of 56 sampled eggs (80%).

4. Discussion

4.1 Habitat selection The water flow through oviposition sites is heavily slowed down by tree roots and stones, forming small cavities (Figure 3 A, C). Eggs were scattered in a monolayer close to the water surface (6–10 cm), which may maximize oxygen exchange for embryo development in the stream (Seymour and Bradford, 1995; Wells, 2007). Some females may deposit eggs beneath huge rocks (> 1 m in diameter) or rock walls in pristine habitat like transect D. Unfortunately, we could not adequately survey these places for the presence of eggs without overturning such rocks.

Nearly all adults sheltered beneath big rocks in the main stream during the daytime, indicating that this species inhabits a very specialized habitat. This may be the reason why the encounter rate in the main stream and another tributary E, which have been heavily quarried, is lower than in the undisturbed tributary D (Figure 2) and why a very small number of egg masses (n =3) were found in the long transect (7.1 km) in the main stream.

4.2 Mating system Among anuran amphibians, two forms of polyandry have been identified according to the time of eggs being fertilized: simultaneous and sequential (Roberts and Byrne, 2011; Byrne and Roberts, 2012). The former arises when sperm ejaculated by more than one male are present at the same time to fertilize eggs, and the latter occurs when a female mates with a series of males in relatively long intervals of time with no risk of sperm competition (Zhang *et al.*, 2012). In this study, we provided the genetic evidence that two females were group-spawning, with three males fertilizing each female's eggs, indicating that this species exhibits simultaneous polyandry, which is similar to the behavior of its sister species *F. taihangnica* (Zhang *et al.*, 2012).

Polyandrous mating systems are commonly associated with high male bias at breeding sites, suggesting polyandry may be a facultative response to male–male competition (Roberts and Byrne, 2011). In contrast to the many examples of facultative polyandry, the operational sex ratio of *F. kangxianensis* is slightly biased to females (59♀:44♂) in the breeding season, which is different with its sister species *F. taihangnica* (190♀:86♂, Chen *et al.*, 2011). The dominant male *F. kangxianensis* fertilized about 85% eggs of Female 1 but only 67% eggs of Female 2, which may support the hypothesis that polyandry may be obligate to increase the fertilization rate of large aquatic egg clutches for females, and (or) to reduce the risk of missing annual reproductive opportunities for

Figure 3 Ovipositon sites, indicated by red arrows, were beneath the bottom of flat stones, in the middle of stream (A, B) or attached to the bank (C). Photo by Jie WANG.

Table 1 Characteristics of the genotypes of nine microsatellite loci that screened in 37 females and 35 males of *F. kangxianensis*.

Locus	Size of alleles	*A*	H_E	H_O	*NE-1P*	*NE-2P*	*NE-PP*	*NE-I*	*NE-SI*
CIBfq01	212–256	12	0.90	0.94	0.37	0.22	0.08	0.02	0.31
CIBfq03	110–158	14	0.70	0.34	0.71	0.53	0.34	0.14	0.44
CIBfq04	377–489	22	0.92	0.93	0.29	0.17	0.05	0.01	0.30
CIBfq05	63–147	18	0.91	0.90	0.32	0.19	0.06	0.02	0.30
CIBfq06	227–251	10	0.38	0.39	0.92	0.78	0.63	0.41	0.67
CIBfq07	168–208	11	0.88	0.86	0.40	0.25	0.10	0.03	0.32
CIBfq08	259–291	9	0.84	0.83	0.51	0.34	0.16	0.05	0.35
CIBfq17	163–165	2	0.35	0.42	0.94	0.86	0.77	0.49	0.70
CIBfq21	232–272	10	0.86	0.83	0.45	0.28	0.12	0.04	0.33
Combined		12	0.75	0.72	1.9×10^{-3}	6.0×10^{-5}			6.3×10^{-12}

Note: Number of alleles (*A*), expected (H_E) and observed (H_O) heterozygosity, non-exclusion probability of one candidate parent (*NE-1P*) or one candidate parent given the genotype of a known parent of the opposite sex (*NE-2P*) or a candidate parent pair (*NE-PP*), non-exclusion probability for identity of two unrelated individuals (*NE-I*) or of two siblings (*NE-SI*).

Table 2 Sibship clustering results using COLONY program based on the genotypes of nine polymorphic microsatellite loci in 56 embryos that collected from one egg mass (containing 503 eggs).

Inferred mother	Embryos from Inferred father			Total embryos
	M1	M2	M3	
FM1	35	5	1	41
FM2	10	4	1	15
Total embryos	45	9	2	

males in explosive breeding species (Zhang *et al.*, 2012).

More detailed studies are needed to understand the evolution of the mating model displayed by *F. kangxianensis* and *F. quandranus* both in sympatry and allopatry. In future investigations, several interesting questions should be addressed in *F. kangxianensis*: How does the dominance in fertilization success link with phenotypic traits and sperm competition? What mode of communication is applied by either sex to achieve aggregation for breeding?

4.3 Species conservation *Feirana kangxianenesis* is recommended to be listed as vulnerable for the IUCN Red List because this species is in significant decline due to habitat degradation and over harvesting (Fei *et al.*, 2012). In this study, we found a high dependence of this species on big stones for shelter and oviposition. However, rural

infrastructure construction is one of the needs of China's modernization and lots of townships and high-class roads are being constructed. More than half of the available stones had been moved away from our study stream to fill the ground base of roads or houses. Meanwhile, sand had been quarried for the stone or brick concretion. A large proportion of the stream is being concreted to reduce bank erosion or to facilitate irrigation. Moreover, the normal runoff decreased or altered after the original riverbeds were destroyed. These activities obviously reduced the specialized habitats and breeding sites of *F. kangxianensis*, thus, habitat conservation, especially putting an end to quarrying, is needed for the persistence of this range-restricted species.

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